

AMENDMENTS

In the Specification:

Please substitute the following for the paragraph beginning on page 1, line 7 and ending on line 24

The present invention relates to a transgenic non-human animal whose germ cells and somatic cells contain a knockout mutation in DNA encoding orphan nuclear receptor $ERR\alpha$. More particularly, the invention relates to a non-human transgenic mammal whose germ cells and somatic cells contain a knockout mutation in DNA encoding orphan nuclear receptor $ERR\alpha$ and more specifically to a transgenic mice whose germ cells and somatic cells contain a knockout mutation in DNA encoding orphan nuclear receptor $ERR\alpha$. In one particular embodiment, mice containing a disruption of both copies of the $ERR\alpha$ gene lack detectable expression of the $ERR\alpha$ protein. The invention further relates to such knockout non-human animals which express an $Err\alpha$ gene which is different from the endogenous gene which was disrupted. In a particular embodiment, the invention relates to a transgenic mouse having its endogenous $ERR\alpha$ gene disrupted and expressing human $ERR\alpha$. As well, the invention relates to cell lines in which $ERR\alpha$ activity (and/or level) has been inactivated or augmented. The invention further relates to uses and methods of the transgenic animals of the present invention to select agents which modulate the expression and/or activity of $ERR\alpha$ and to agents identified by these methods.

Please substitute the following for the paragraph beginning on page 2, line 16 and ending on page 3, line 6

Obesity is a prevalent disorder that often leads to diabetes, cardiovascular disease, and joint disorders. Although the precise mechanism which leads to the development of obesity has yet to be precisely determined, it appears clear that a number of mechanisms, which normally function to maintain homeostasy and normal body weight are involved. Transgenic mice with an induced brown fat deficiency have indicated that this tissue is implicated in the control of the balance of in mice (Lowell et al., Nature 366:740-742, 1993). Further, a correlation between brown adipose tissue dysfunction and obesity and diabetes has been reported (Lowell et al., *Supra*). Previous studies have demonstrated that $ERR\alpha$ is highly expressed in brown adipose tissue (BAT)

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during murine development and that the receptor is upregulated during white and brown adipocyte differentiation *in vitro* (Sladek et al., 1997; Vega and Kelly, 1997). In addition, ERR α has been shown to modulate the activity of the medium chain acyl-coA dehydrogenase (MCAD) promoter, a key regulatory step in the fatty acid β -oxidation pathway (Sladek et al., 1997; Vega and Kelly, 1997). More recently, a transgenic mouse whose germ cells and somatic cells contain a knockout mutation in DNA encoding an endogenous beta.₃-adrenergic receptor polypeptide, thereby obtaining a mouse having a modest increase in body fat, has been reported (U.S. 5,789,654).

Please substitute the following for the paragraph beginning on page 8, line 19 and ending on page 9, line 9

It will also be apparent that the cells and tissues of the transgenic animals of the present invention can be useful in *in vitro* methods relating to fat deposition and related disorders (including rational design and/or screening of compounds which can modulate expression and/or activity of the ERR α orphan nuclear receptor. In a related aspect, the present invention further relates to cell lines in which the activity of ERR α has been inactivated or augmented. In addition to being derived from the transgenic animals of the present invention, such cell lines, can for example be derived as commonly known in the art using the construct of the present invention or derivatives or variants thereof. Such cell lines can be used similarly to the animals of the present invention to identify compounds which modulate ERR α level and/or activity, dissect the physiological and biochemical function (including structure/function relationships, as they relate to fat deposition and the like) of ERR α . Thus, the present invention also relates to established cell lines or primary cells derived from an animal of the present invention. In one embodiment, fat pads from a transgenic mouse of the present invention was used to obtain primary cells which were grown and used in *in vitro* methods (i.e. insulin effect, glucose uptake, lipogenesis measurements and the like). Such experiments validated these cells as a pertinent tool for the methods and uses of the present invention.

Please substitute the following for the paragraph beginning on page 11, line 10 and ending on line 11

(c) transplanting the animal zygote into a pseudopregnant compatible animal;

Please substitute the following for the paragraph beginning on page 13, line 11

iii) AGG TCA NNN TGA CCT (SEQ ID NO:1); and

Please substitute the following for the paragraph beginning on page 14, line 17

iii) AGG TCA NNN TGA CCT (SEQ ID NO:1); and

Please substitute the following for the paragraph beginning on page 15, line 17 and ending on page 16, line 2

As used herein, "non-human transgenic animal" is any non-human animal in which at least one cell comprises genetically altered information through known means such as microinjection, virus-delivered infection, or homologous recombination. In one particularly preferred embodiment of the present invention, the transgenic animal is a transgenic mouse, in which the genetic alteration has been introduced in a germ-line cell such, that it enables the transfer of this genetic alteration to the offsprings thereof. Such offsprings, containing this genetic alteration, are also transgenic mice.

Please substitute the following for the paragraph beginning on page 16, line 23 and ending on page 17, line 6

The terminology "estrogen response elements" or "estrogen cis-acting elements" refers to well-known nucleic acid sequences to which transcription factors such as the orphan nuclear receptor $ERR\alpha$ can bind, thereby having the potential to modulate the promoter activity of a promoter comprising such response or cis-acting elements. These cis-acting elements or estrogen response elements also termed "ERE" or "IR3" are well-known in the art (Petterson, 1996, Mech. Dev. 54:211-223). In Petterson et al. (1996, *supra*), it is for example taught that the perfect inverted repeat (IR) of the estrogen response element to which $ERR\alpha$ can bind has sequence AGG TCA NNN TGA CCT (SEQ ID NO:1). It is also known from Sladek et al., 1997, Bonnelye et al., 1997 and Johnston et al., 1997 that this acting element comprising the sequence TGA AGG TCA can also bind $ERR\alpha$ and related factors.

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Please substitute the following for the paragraph beginning on page 22, line 3 and ending on line 20

Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. patents 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosures of all four U.S. patents are incorporated herein by reference). In general, PCR involves, a treatment of a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) under hybridizing conditions, with one oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of each primer which is synthesized is complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products using the same primers. Following a sufficient number of rounds of synthesis of extension products, the sample is analysed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization following EtBr staining of the DNA following gel electrophores, or using a detectable label in accordance with known techniques, and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al. Eds, Acad. Press, 1990).

Please substitute the following for the paragraph beginning on page 26, line 11 and ending on line 13:

The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. All these methods are well known in the art.

Please substitute the following for the paragraph beginning on page 28, line 24 and ending on page 29, line 4

As well, having identified $ERR\alpha$ as a target for lipogenesis, fatty acid esterification and fatty acid oxydation modulation, $ERR\alpha$ can be used in a number of *in vitro* and *in vivo* assays to identify ligands therefor and dissect its structure/function relationship. Non limiting examples thereof include binding assays and the two hybrid system technology, as well known in the art (Ausubel et al., 1994, supra). This assay has proven beneficial to test compounds or a library thereof. Thus, the invention also covers $ERR\alpha$ -expressing cells (prokaryotes, lower and higher

eukaryotes) or variants thereof to identify mutations which modulate ERR α activity or compounds which have ERR α modulating effects.

Please substitute the following for the paragraph beginning on page 29, line 5 and ending on line 20

The present invention also provides antisense nucleic acid molecules which can be used for example to decrease or abrogate the expression of the nucleic acid sequences or proteins of the present invention. An antisense nucleic acid molecule according to the present invention refers to a molecule capable of forming a stable duplex or triplex with a portion of its targeted nucleic acid sequence (DNA or RNA). The use of antisense nucleic acid molecules and the design and modification of such molecules is well known in the art as described for example in WO 96/32966, WO 96/11266, WO 94/15646, WO 93/08845 and U.S. patent 5,593,974. Antisense nucleic acid molecules according to the present invention can be derived from the nucleic acid sequences and modified in accordance to well known methods. For example, some antisense molecules can be designed to be more resistant to degradation to increase their affinity to their targeted sequence, to affect their transport to chosen cell types or cell compartments, and/or to enhance their lipid solubility by using nucleotide analogs and/or substituting chosen chemical fragments thereof, as commonly known in the art.

Please substitute the following for the paragraph beginning on page 34, line 17 and ending on page 35, line 6

Figs. 1A-1D show the targeted disruption of the *Estrra* gene and heterozygote inbreeding analysis. Fig. 1A, Structure of the ERR α locus, targeting vector, and recombinant allele. Top, map of the wild type locus: exons are indicated by black boxes. E2 encodes the upstream zinc-binding motif of the ERR α DNA-binding domain. Center, targeting construct. Bottom, map of the targeted allele, showing replacement of exon 2 sequences by the *neo*^r cassette. The restriction enzyme digests and the probes used to characterize the knockout mice are illustrated. B, *Bam*HI; H, *Hind*III. Fig. 1B, Southern blot analysis of targeted ES clones. DNA from parental ES cells (R1) and two targeted clones (57 and 62) was digested with *Bam*HI and hybridized to the 3' probe. The positions of bands corresponding to the wild-type (10.7 kb) and targeted alleles (4.5 kb) are indicated (upper panel). Single integration of the targeting construct in targeted ES cell

clones was confirmed with a *neo*^r probe: a single hybridizing band (6.0 kb) is present in the targeted lines (lower panel). Fig. 1C, Southern blot analysis of genotypes of 28d old pups from a heterozygote intercross: the litter contains viable homozygous null mice. Fig. 1D, Northern blot analysis of RNA obtained from the kidneys of the progeny of heterozygous intercrosses. *ERRα* expression is not detected in RNA samples obtained from homozygous null mutants.

Please substitute the following for the paragraph beginning on page 35, line 7 and ending on line 16

Figs. 2A-2D show the phenotypic analysis of *Estrra* null mutants. Fig. 2A, Mutant animals display decreased weight gain. Growth curves were performed by weighing animals at the indicated ages: both male and female knockout mice display significantly reduced body weight in comparison to their wild-type littermates. Arrows indicate start of pre-pubertal growth spurt. Fig. 2B, Body composition of *Estrra* null mice shows decreased ratio of fat to lean mass. Fig. 2C, *Estrra*^{-/-} mice contain decreased body fat. Superficial carcass dissection of two 20 week old male mice shows the decreased body fat content of a 32.9 g knockout mouse (right) in comparison with his 38.1 g wild-type littermate (left). Fig. 2D, The difference in body composition is reflected by the relative sizes of the dissected fat pads.

Please substitute the following for the paragraph beginning on page 35, line 17 and ending on line 24

Figs. 3A-3C show the analysis of intestinal lipid transport in *Estrra* null mutants. Fig. 3A, Thin layer chromatographic analysis of tissue lipid content. The intestines of *Estrra*^{-/-} mice contain decreased triglyceride and increased free fatty acids in comparison with their wild-type and heterozygous littermates. Fig. 3B, Analysis of glycerolipid synthesis in *Estrra* null mutants. *Estrra*^{-/-} mice demonstrate reduced triglyceride synthesis in intestinal and hepatic whole cell extracts. Fig. 3C, Fat absorption profile. *Estrra*^{-/-} mice and littermate controls display similar rates of absorption of radiolabeled oleic acid.

Please substitute the following for the paragraph beginning on page 35, line 25 and ending on page 36, line 2

Figs. 4A and 4B show the analysis of adipocyte function in *Estrra* null mutants. Fig. 4A, Histologic studies of epididymal fat pads show that *Estrra*^{-/-} mice (lower panel) have decreased adipocyte volume in comparison to wild-type animals (upper panel). Fig. 4B, *Estrra*^{-/-} mice demonstrate decreased lipogenesis in comparison to littermate controls.

Please substitute the following for the paragraph beginning on page 36, line 13 and ending on page 37, line 3

The method of production and the transgenic animals of the present invention are described herein below. In general, these animals are produced by engineering a nucleic acid construct which can disrupt the expression of the endogenous $ERR\alpha$ gene (i.e., the murine $ERR\alpha$ gene). Using known methods, this construct is amplified in bacterial cells, purified, and transferred into ES cells or isolated oocytes. The transfected ES cells can then be injected into blastocysts to generate chimeras. The chimeras which transmit the mutation to their offspring are identified and selected. These animals can then be used as founder animals to obtain different animal lines, derived from breeding with chosen animals. Heterozygous animals can then be produced and further mated to generate a hybrid F1 cross. Further matings of the F1 heterozygotes produce the wild type, heterozygous and homozygous null mutants of $ERR\alpha$ (having both copies of the $ERR\alpha$ gene disrupted). The homozygous animals can then serve in a number of experiments. Non-limiting examples thereof include: the characterization of their phenotype, and a reconstitution of the $ERR\alpha$ activity by complementation by a non-endogenous copy of a wild type $ERR\alpha$ gene or mutant or variant $ERR\alpha$ gene. An animal (or cells derived therefrom) expressing a mutant form of $ERR\alpha$ gene (from human, for example) could be used to screen for compounds which modulate more specifically the mutant form of the $ERR\alpha$ gene.

Please substitute the following for the paragraph beginning on page 37, line 4 and ending on line 11

The present invention therefore strongly indicates that $ERR\alpha$ is a direct regulator of fundamental cellular function. It is thus expected that this cellular function should occur across species. The presence of the $ERR\alpha$ gene and its conservation among species (human, mice, rats,

fish and lower organisms; Escriva et al. (1997) Proc. Natl. Acad. Sci. USA 94:6803-6808), support its essential role in physiology. Thus, the antagonists identified by the methods and assays of the present invention should find a utility in the treatment of obesity and other metabolic diseases associated with $ERR\alpha$ malfunction in humans.

Please substitute the following for the paragraph beginning on page 37, line 12 and ending on line 13

The present invention is illustrated in further detail by the following non-limiting examples.

Please substitute the following for the paragraph beginning on page 37, line 17 and ending on page 38, line 8

Three overlapping λ clones containing the mouse *Estrra* locus were isolated from a 129Sv genomic library (gift of Dr. A. Joyner, Skirball Institute, New York) and characterized by restriction mapping and direct sequencing of the exon boundaries. The knockout construct was created using pNT (Tybulewicz et al., 1991) and contained 6.4 Kb of genomic DNA flanking the second exon of *Estrra*. An endfilled 4.2 Kb *Bam*HI/*Not*I fragment, lying upstream of the second exon, was cloned into the *Xho*I site of pNT, while a 2.2 Kb *Hind*III fragment was cloned between the *neor* and TK cassettes to provide the 3' arm of the construct. Correct targeting of the *Estrra* locus replaces the receptor's second exon, which encodes a critical part of its DNA binding domain, with a *neo* cassette. The linearized construct was electroporated into R1 ES cells (Nagy and Rossant, 1993) which were selected with G418 (150 μ g/mL) and gancyclovir (2 μ M). Two ES cell clones were isolated and injected into C57BL6 blastocysts to generate chimeras, and three chimeras transmitted the mutation to their offspring. Heterozygous mice, generated by mating the chimeric animals with 129SvJ mice were mated with C57BL6 animals to generate hybrid F1 animals: physiologic studies were performed using the F2 null mutant and wild-type offspring obtained by mating the F1 hybrid heterozygotes. Complete disruption of the *Estrra* allele was verified by performing Northern blots using RNA obtained from placenta and kidneys of homozygous mutants.

Please substitute the following for the paragraph beginning on page 39, line 23 and ending on page 40, line 3

Mice were studied at 10:00h following free access to food overnight. The animals were conditioned by sham intraperitoneal injections of water. On the day of the experiment, the animals were injected intraperitoneally with $^3\text{H}_2\text{O}$ (0.5 mCi per 100 g body weight) and sacrificed by cervical dislocation 30 minutes later. Serum, adipose tissue and liver samples were harvested and stored at -80°C . The tissues were homogenized and heated in ethanolic KOH: the resulting extract, which contained saponified lipids, was acidified using concentrated sulfuric acid and extracted using petroleum ether. The extract was dried by evaporation and ^3H incorporation determined by scintillation counting.

Please substitute the following for the paragraph beginning on page 44, line 6 and ending on line 24

Previous studies have demonstrated that $\text{ERR}\alpha$ is highly expressed in brown adipose tissue (BAT) during murine development and that the receptor is upregulated during white and brown adipocyte differentiation *in vitro* (Sladek et al., 1997; Vega and Kelly, 1997). In addition, $\text{ERR}\alpha$ has been shown to modulate the activity of the medium chain acyl-coA dehydrogenase (MCAD) promoter, a key regulatory step in the fatty acid β -oxidation pathway (Sladek et al., 1997; Vega and Kelly, 1997). As dysregulation of BAT function has been associated with abnormalities of body composition, therefore, a characterization of BAT function in the $\text{ERR}\alpha$ knockout mice was carried out. $\text{ERR}\alpha$ null mutants had normal core body temperature and basal metabolic rate and displayed normal expression levels of uncoupling protein (UCP) mRNA in BAT (UCP-1) and skeletal muscle (UCP-2) (data not shown). Defects in fatty acid oxidation are frequently only apparent following situations of physiologic stress or food deprivation: neither prolonged cold exposure or fasts of up to 48 hour's duration resulted in any morbidity or mortality of $\text{Estr}\alpha^{-/-}$ mice (data not shown). Taken together, these data suggest that the abnormal body composition seen in $\text{ERR}\alpha$ null mutants was not a result of increased thermogenesis or increased basal energy expenditure, and that the animals did not have physiologically significant defects in fatty acid β -oxidation.

Please substitute the following for the paragraph beginning on page 44, line 25 and ending on page 45, line 15

Fat pads obtained from *Estrra* mutants displayed decreased adipocyte volume in comparison to wild-type animals (Fig. 4A), which suggests that the decreased adipose tissue mass observed in *Estrra*^{-/-} mice results from an imbalance between fatty acid synthesis and lipolysis rather than defects in adipocyte proliferation and differentiation. As *ERRα* expression is induced during early adipocyte differentiation *in vitro* (Sladek et al., 1997), it is possible that *ERRα* acts as a regulator of processes important for adipocyte function, such as fatty acid synthesis or esterification. In animals fed a standard laboratory diet, murine adipose tissue contains triglyceride formed from fatty acids that are synthesized *de novo* rather than from dietary lipid. Lipogenesis was assessed by treating *Estrra*^{-/-} mice with ³H₂O: the amount of radioactive label incorporated into triacylglycerol can be measured by saponification and ether extraction of adipose tissues and other organs. *Estrra* null mutants demonstrate significantly decreased lipogenesis in comparison to littermate controls: in particular, knockout animals show a 30-55% decrease in ³H incorporation into adipose tissue lipids and a 50% decrease in ³H incorporation into hepatic lipids (Fig. 4B). This observation demonstrates that adipose tissue of knockout mice possesses a defect in TG synthesis, which may result from decreased adipocyte and hepatic glycolysis activity, fatty acid synthesis or esterification.

Please substitute the following for the paragraph beginning on page 45, line 16 and ending on page 46, line 12

Experiments performed using the *Estrra*^{-/-} mice revealed that *ERRα* is a key regulator of fat metabolism, including intestinal fat transfer and esterification, as well as hepatic and adipocyte fat deposition. *Estrra*^{-/-} mice display decreased fat content associated with reduced intestinal fatty acid esterification rates and abnormal regulation of fat deposition and mobilization in adipocytes and liver. Previous *in vitro* studies have demonstrated that *ERRα* modulates the expression of MCAD, a key regulatory enzyme of fatty acid β -oxidation, a pathway which may also play a role in establishing the *ERRα* phenotype. The relative importance of each of these effects in establishing the body composition of *ERRα* mice remains to be determined. Since the *Estrra*^{-/-} mice show a normal level of energy intake, one would expect to observe an increase in energy expenditure to account for the decreased fat content of these mice. However, the sensitivity